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Dihydrooxadiazines: Octopaminergic System as a Potential Site of Insecticidal Action

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Abstract: Dihydrooxadiazines are structural analogs of octopamine and were compared with octopamine for their ability to compete with [^{3}H]dihydroergocryptine ([^{3}H]DHE) for binding sites on DHE-sensitive receptors, to stimulate adenylate cyclase activity in nervous system homogenates of *Periplaneta americana* L., and to modulate the action of the peptide proctolin on the oviducal muscles of *Locusta migratoria* L. [^{3}H]DHE binding was inhibited by low concentrations (μM range) of octopamine, phentolamine, *N*-demethylchlordimeform (DCDM) and several dihydrooxadiazines. The tested dihydrooxadiazines acted as aminergic agonists in stimulating cyclic AMP production in cockroach nervous system homogenates and did not show additive effects with octopamine, whereas additivity was observed with 5-hydroxytryptamine. The relative potency of octopaminergic antagonists, including mianserin, cyproheptadine, phentolamine, and gramine, to block octopamine-mediated elevation of cyclic AMP production was similar to the rank-order potency of the same antagonists to inhibit dihydrooxadiazine-mediated elevation of cyclic AMP production. Octopamine, 2-(4-bromophenyl)-5,6-dihydro-4*H*-1,3,4-oxadiazine (4-Br-PDHO), and 8-Br-cyclic AMP caused increased phosphorylation of proteins that are phosphorylated by exogenously added cyclic AMP-dependent protein kinase. These results indicate that the dihydrooxadiazine-induced rise in cyclic AMP levels in homogenates of the cockroach nervous system results directly in activation of an endogenous cyclic AMP-dependent protein kinase. 4-Br-PDHO behaved similarly to octopamine in modulating the action of proctolin-induced contractions in locust oviducal muscles. These observations suggest that dihydrooxadiazines act as octopamine agonists and have an octopaminergic action in modulating the action of proctolin. Thus, it is proposed that dihydrooxadiazines exert at least part of their insecticidal and miticidal actions through interaction with the octopaminergic system.

Key words: dihydrooxadiazines, octopamine agonists.

1 INTRODUCTION

Many physiological processes in insects are regulated by the monoamines, octopamine, dopamine and 5-hydroxytryptamine.[1] In some cases, the aminergic effects are expressed through interaction of the amine with a specific membrane receptor that is coupled to the adenylate cyclase system.[2] Binding of the amine to the adenylate cyclase-coupled receptor results in elevated intracellular levels of adenosine 3′,5′-phosphate (cyclic AMP)[1] and subsequent phosphorylation of target proteins.[3,4]

Several classes of insecticide appear to exert their actions through perturbation of aminergic interaction

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with the specific receptor protein, including formamidines,[5] imidazolines,[6] oxazolines,[7] and thiazolines.[8] Indeed, in that the pharmacology of monoaminergic receptors in insects appears to be distinct from that of reported aminergic receptors in vertebrates,[1] there is much potential for exploration of insect aminergic receptors in the development of highly specific insecticides.

5,6-Dihydro-4*H*-1,3,4-oxadiazines are structural analogs of octopamine with insecticidal and miticidal activity.[9-11] These compounds elevate cyclic AMP levels in homogenates of two-spotted spider mites, *Tetranychus urticae* Koch,[12] and the increased cyclic AMP production is inhibited by several octopaminergic antagonists, including mianserin, cyproheptadine, phentolamine and gramine. Octopamine, 2-(4-bromophenyl)-5,6-dihydro-4*H*-1,3,4-oxadiazine (4-Br-PDHO), and 8-Br-cyclic AMP also increase the phosphorylation of proteins that are phosphorylated by exogenous cyclic AMP-dependent protein kinase. These observations suggest that dihydrooxadiazines may exert their insecticidal and miticidal actions through perturbation of the octopaminergic system in mites and the primary site of dihydrooxadiazine action may be the octopamine-sensitive adenylate cyclase complex. The present study investigates the effects of dihydrooxadiazines on [^{3}H]dihydroergocryptine ([^{3}H]DHE) binding sites and on cyclic AMP production in the nervous system homogenates of the American cockroach, *Periplaneta americana* L., and consequent effects on protein phosphorylation. The ergot alkaloid, dihydroergocryptine, has been used to screen for aminergic receptors in insects.[13] In addition, the study compares the relative effectiveness of dihydrooxadiazines and octopamine in modulating the actions of proctolin on oviducal musculature in the locust, *Locusta migratoria* L., as demonstrated by Lange *et al.*,[14] and Baines and Downer.[15]

2 MATERIALS AND METHODS

2.1 Insects

Adult male cockroaches were taken from a colony of *P. americana* maintained in the laboratory for several generations on Purina Dog Chow and water under a 12 : 12 h light : dark cycle.[16] Locusts were obtained from a colony of *L. migratoria* maintained in the laboratory under constant illumination and fed wheat seedlings and bran.

2.2 Chemical synthesis

The chemical structures of the dihydrooxadiazine compounds used in this study are shown in Fig. 1. All compounds were prepared as their hydrochloride salts.

2.2.1 Preparation of 4-Br-PDHO hydrochloride (**1**)
A solution of sodium hydroxide (2·0 g; 0·04 mol) in water (10 ml) was added dropwise to a stirred mixture of 4-bromobenzhydrazide (4·3 g; 0·02 mol) and 1-bromo-2-fluoroethane (2·5 g; 0·02 mol) in ethanol (50 ml). The reaction mixture was heated to reflux for 2 h. After cooling to room temperature, the reaction mixture was poured into water (200 ml) and extracted with diethyl ether (3 × 100 ml). The ether extracts were combined, dried over sodium sulfate and then evaporated leaving an oil. The oil was purified by column chromatography on silica gel by eluting with ethyl acetate + dichloromethane (1 + 1 by volume). NMR

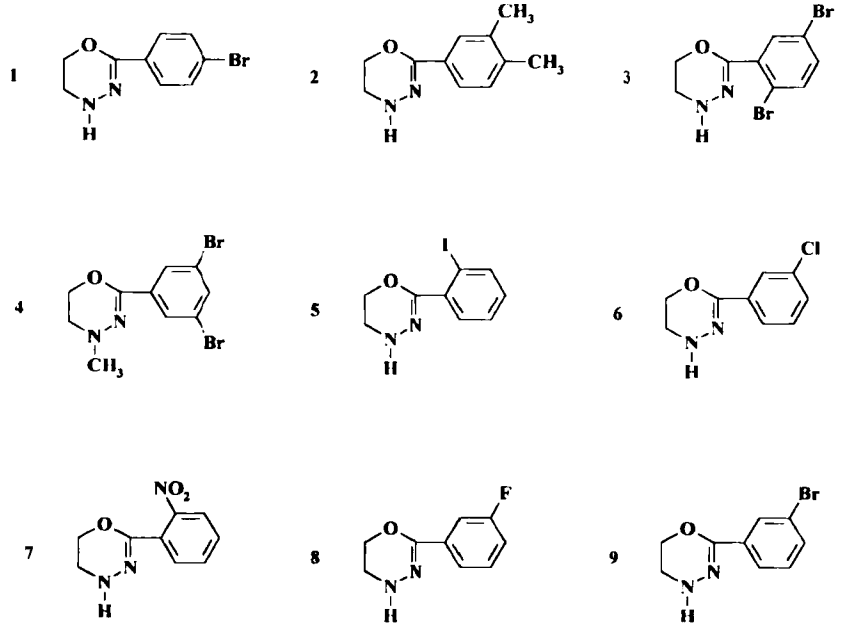

Fig. 1. Structures of dihydrooxadiazine compounds **1**–**9** used in this study.

showed the purified oil to be the dihydrooxadiazine. The resulting oil was dissolved in toluene and treated dropwise with a saturated solution of hydrogen chloride in toluene until no further precipitate appeared. The precipitated solid was collected, washed with toluene and air-dried. NMR showed this to be the hydrochloride of the dihydrooxadiazine, with a melting point of 165–168°C. Anal. calc. for $C_9H_{10}BrClN_2O$: C, 39·00; H, 3·61; N, 10·10; Found: C, 39·48; H, 3·32; N, 9·98.

The other 5,6-dihydro-4*H*-1,3,4-oxadiazines reported in this work and prepared using the methods described above are the 3,4-dimethylphenyl (**2**) (m.p. 144–145°C), 2,5-dibromophenyl (**3**) (m.p. 159–161°C), 2-iodophenyl (**5**) (m.p. 138–142°C), 3-chlorophenyl (**6**) (m.p. 152–155°C), 2-nitrophenyl (**7**) (m.p. 118–121°C), 3-fluorophenyl (**8**) (m.p. 143–145°C) and 3-bromophenyl (**9**) (m.p. 183–184°C) derivatives, respectively.

2.2.2 Preparation of 2-(3,5-dibromophenyl)-5,6-dihydro-4-methyl-4H-1,3,4-oxadiazine hydrochloride (**4**)

A mixture of methyl 3,5-dibromobenzoate (3·8 g; 0·02 mol) and methyl hydrazine (2·3 g; 0·05 mol) was refluxed for 24 h, then evaporated leaving the 2-methyl hydrazide in a relatively pure state. The 2-methyl hydrazide (6·0 g; 0·02 mol) was dissolved in ethanol (50 ml), combined with 1-bromo-2-fluoroethane (2·5 g; 0·02 mol), and treated dropwise with a solution of sodium hydroxide (2·0 g; 0·04 mol) in water (10 ml). The resulting mixture was refluxed for 2 h, cooled to room temperature, and poured into water (200 ml). The product was extracted with diethyl ether(3 × 100 ml), dried over sodium sulfate and evaporated, leaving the final product essentially pure, as identified by NMR. The resulting oil was converted into its hydrochloride salt, as described above, with a melting point of 195–198°C. Anal. calc. for $C_{10}H_{11}Br_2ClN_2O$: C, 32·43; H, 3·00; N, 7·56; Found: C, 32·64; H, 3·16; N, 8·00.

2.3 Chemicals

[^{3}H]Dihydroergocryptine (20 Ci mmol^{-1}), [^{125}I]-cyclic AMP (5 μCi) and cyclic AMP antibody were purchased from DuPont (Boston, MA). *N*-Demethylchlordimeform (DCDM) was provided by Dr I. Orchard, University of Toronto, and phentolamine by Ciba-Geigy (Dorval, Quebec). Other drugs were purchased from Sigma Chemical Co. (St. Louis, MO).

2.4 [^{3}H]Dihydroergocryptine binding assay

The nervous system (brain and ventral nerve cord) was dissected from 300–400 adult male cockroaches on an ice bath, taking care to remove traces of fat body and integument. The tissues were frozen and stored at −80°C for a maximum of one week before use. The frozen tissues were homogenized in 20 volumes of ice-cold 2-hydroxy-1,1-bis(hydroxymethyl)ethylammonium chloride (Tris) buffer (50 mM, pH 7·4) containing 5 mM magnesium chloride, using a glass/Teflon homogenizer. The homogenates were centrifuged at 500 *g* for 10 min to pellet the crude nuclear fraction. The supernatant was centrifuged twice at 50 000 *g* for 15 min at 4°C with intermediate resuspension. The third supernatant fraction was carefully decanted and the final pellet resuspended in the same Tris buffer at 1·1–1·3 mg tissue ml^{-1}. The suspension was stored frozen in 1-ml aliquots in 1·5-ml sealed plastic test tubes at − 80°C. Before use, the suspension was thawed and recentrifuged at 50 000 *g* for 1 h. The pellets were then resuspended in the original volume of the same Tris buffer.

Tissue binding experiments with [^{3}H]DHE were conducted in 10 × 130-mm culture tubes at 27°C in a shaking water bath. Each tube received 95–100 μl of the washed membrane protein (120–125 μg protein per assay tube), 100 μl of unlabeled test chemicals, 38–40 μl of [^{3}H]DHE to give a final concentration of 2 nM [^{3}H]DHE, and 753–760 μl of 50 mM Tris—HCl containing 5 mM magnesium chloride and 2 mM ascorbic acid, pH 7·4, in a volume of 1 ml. Each treatment was triplicated. The [^{3}H]DHE was dissolved in the same Tris/magnesium chloride/ascorbic acid buffer pH 7·4. The unlabeled drugs were dissolved in 2 ml litre^{-1} dimethyl sulfoxide (DMSO) in Tris/magnesium chloride/ascorbic acid buffer.

The reaction was started by the addition of [^{3}H]DHE, continued for 30 min at 27°C and terminated by 10-fold dilution of the reaction mixture with ice-cold Tris-HCl buffer (pH 7·4) containing 5 mM magnesium chloride followed immediately by vacuum filtration through a pre-rinsed Whatman GF/C glass-fiber filter. The filters were immediately rinsed three times with a 5-ml aliquot of the same Tris—HCl buffer, and placed in plastic vials for scintillation counting.

2.5 Cyclic AMP assay

The procedures used for measuring cyclic AMP levels in the nervous system (brain and nerve cord) homogenates of the American cockroach were essentially those developed by Downer *et al.*[17] Freshly dissected brains and nerve cords were homogenized in an ice-cold solution containing 10 mM Tris-acetate, 1 mM ethylenediamine tetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT), pH 7·0, using a glass/Teflon homogenizer. The homogenate was then centrifuged at 28 000 *g* for 20 min at 4°C, the pellet resuspended in 10 ml of Tris—DTT, pH 7·0, recentrifuged and the pellet again resuspended in Tris—DTT, pH 7·0. Enzyme activity was measured following incubation of 100 μl of homogenate in a medium containing 75 mM Tris-acetate, 0·1 mM 3-isobutyl-1-methylxanthine (IBMX), 10 mM magnesium acetate, 50 μM guanosine triphosphate (GTP) and 2·5 mM adenosine triphosphate (ATP) in a final volume of 200 μl. The test

compound was added with 20 μl of 20 ml litre^{-1} DMSO to give a final concentration of 2 ml litre^{-1} DMSO in the final volume of the reaction mixture. The reaction was started by the addition of ATP. The mixture was incubated for 5 min at 30°C in a shaking water bath and then placed in an ice-cold water bath. The reaction was stopped by the addition of 1 ml of perchloric acid (0·4 M). After 10 min at 30°C, the acid was neutralized by the addition of 200 μl of potassium hydrogen carbonate (2·5 M). Following centrifugation at 1500 *g* for 5 min, an aliquot of the supernatant was assayed for cyclic AMP by radioimmunoassay using the 'RIANEN®' kit (New England Nuclear, Boston, MA). An aliquot of the nervous system suspension was measured for protein content using the Biorad protein assay kit employing human gamma globulin as the standard (Biorad, Mississauga, ON).

2.6 Studies on protein phosphorylation

The procedures adopted were identical to those developed by Costa *et al.*,[18] and Ismail and Matsumura.[3,4] The procedures for cyclic AMP assay were repeated; however, the reaction was stopped by adding 10 g litre^{-1} sodium dodecylsulfate (SDS) (20 μl) and heating the mixture at 100°C for 2 min. On cooling in an ice-water bath, 100 g litre^{-1} 'Triton' X-100 (20 μl) was added and the system thoroughly vortexed, centrifuged at 500 *g* for 5 min at 4°C and the supernatant collected. A 50-μl aliquot of the supernatant was transferred to a small test tube containing 10 μl (600 ng) of catalytic subunit of protein kinase. The [^{32}P]phosphorylation reaction was initiated by the addition of 2 mCi of γ-[^{32}P]ATP (20 ml) in distilled water. After 10 min at 30°C, the reaction was stopped with 80 μl of 2 × 'treatment buffer' 40 g litre^{-1} SDS, 200 g litre^{-1} glycerol, 100 g litre^{-1} 2-mercaptoethanol in 0·125 M Tris—HCl, pH 6·8) and heating to 100°C for 2 min. The entire volume of the reaction product in each tube was transferred to an electrophoresis well. The method of SDS polyacrylamide slab gel electrophoresis used was that of Takacs[19] using a Bio-Rad Protein II System at 30 mA (with a 1·5-mm spacer).

2.7 Physiology

The oviducts of adult female *L. migratoria* were dissected together with ovaries and short lengths of oviducal nerves. The oviducts were attached at one end to a wax dish using small pins and at the other end to a force transducer (Harvard 373 Isometric Transducer) using a fine thread. The preparations were maintained by washing with saline (sodium chloride 128 mM, potassium chloride 10 mM, magnesium chloride 2·5 mM, calcium chloride 2 mM, sodium hydrogen carbonate 2 mM, disodium hydrogen phosphate 2 mM, sodium dihydrogen phosphate 1·5 mM and glucose 55 mM, pH 7·0). Spontaneous and induced contractions were monitored and recorded on a chart recorder (Perkin-Elmer R-100). The preparations were exposed to saline solutions of proctolin (1×10^{-9} M), proctolin (1×10^{-9} M) + octopamine (1×10^{-6} M), phentolamine (1×10^{-4} M), proctolin (1×10^{-9} M) + octopamine (1×10^{-6} M) + phentolamine (1×10^{-4} M), proctolin (1×10^{-9} M) + 4-Br-PDHO (**1**) (1×10^{-6} M), proctolin (1×10^{-9} M) + 4-Br-PDHO (**1**) (1×10^{-6} M) + phentolamine (1×10^{-4} M), alternately washing the preparation with saline until myogenic contractions resumed.

3 RESULTS

Studies with the 50 000 *g* membrane fraction of the American cockroach nervous system routinely demonstrated a specific [^{3}H]DHE binding component. Non-specific binding, defined as the binding observed in the presence of 1×10^{-5} M dihydroergocryptine, was less than 6% under the standard assay condition. Specific binding of [^{3}H]DHE, defined as total binding minus non-specific binding, represented 66% of total binding at 0·1 nM dihydroergocryptine and 52% at 1 nM. The specificity of [^{3}H]DHE binding was examined by testing the ability of various ligands to displace [^{3}H]DHE from its binding sites. The results are presented in Table 1. Log concentration–logit inhibition plots showed a linear relationship in each case. From these plots, the IC_{50} values (concentration of ligand required to inhibit specific DHE binding by 50%) were determined.

Four dihydrooxadiazines displayed half-maximal inhibition at the 10^{-6} M range. These were compounds

TABLE 1

Inhibition of [^{3}H]DHE[a] Binding to Nervous System Preparations of the American Cockroach by Various Ligands

Ligand	IC_{50} (M)
Dihydroergocryptine	$4·1 \times 10^{-9}$
4	$1·0 \times 10^{-6}$
2	$2·0 \times 10^{-6}$
6	$4·0 \times 10^{-6}$
1	$5·0 \times 10^{-6}$
3	$8·0 \times 10^{-5}$
5	$2·0 \times 10^{-4}$
7	$8·0 \times 10^{-4}$
9	$1·0 \times 10^{-3}$
8	$5·0 \times 10^{-3}$
Phentolamine	$7·0 \times 10^{-6}$
Octopamine	$5·0 \times 10^{-6}$
DCDM	$6·0 \times 10^{-5}$
5-Hydroxytryptamine	$4·0 \times 10^{-4}$

[a] Concentration of radioligand [^{3}H]DHE was 2 nM.

1, **2**, **4** and **6** in descending order of potency. Octopamine and phentolamine showed IC_{50} values of 5×10^{-6} M and 7×10^{-6} M, respectively, whereas DCDM and dihydrooxadiazine **3** displayed IC_{50} values in the 10^{-5} M range. 5-Hydroxytryptamine and dihydrooxadiazines **5** and **7** showed IC_{50} values in the 10^{-4} M range. Dihydrooxadiazines **8** and **9** displayed IC_{50} values in the 10^{-3} M range. Thus, several dihydrooxadiazines were more effective than octopamine, phentolamine, 5-hydroxytryptamine, and DCDM in inhibiting the binding of 2 nM [^{3}H]DHE.

The abilities of dihydrooxadiazines to activate or inhibit adenylate cyclase were tested in nervous system homogenates of the American cockroach. The results found (Table 2) show the effects of octopamine, 5-hydroxytryptamine, DCDM and dihydrooxadiazines at 1×10^{-5} M on cyclic AMP levels. Octopamine and DCDM caused the most pronounced elevation in cyclic AMP production with 465% and 308% increases, respectively, over the control value. The dihydrooxadiazines differed in their abilities to stimulate adenylate cyclase activity. Compounds **1**–**3** were the most potent in elevating cyclic AMP production, with 165, 144 and 97% increases, respectively, over the control value. The remaining dihydrooxadiazines increased cyclic AMP production with a range of 31–83% over the control value. The effects of various concentrations of octopamine, 5-hydroxytryptamine and dihydrooxadiazines **1**–**3** on cyclic AMP production are illustrated in Fig. 2. The results indicate a concentration-dependent elevation of cyclic AMP production in the range of 1×10^{-7} M to 1×10^{-4} M.

The possibility that dihydrooxadiazines may interact with the same binding sites for octopamine and 5-hydroxytryptamine in nervous system homogenates of the American cockroach was investigated by examining the additive effects of the three most potent dihydrooxadiazines (compounds **1**–**3**), octopamine, and 5-hydroxytryptamine in elevating cyclic AMP production at maximum concentration. The results shown in Table 3 indicate that the level of evoked cyclic AMP production due to a combination of 100 μM of octopamine and 100 μM of dihydrooxadiazine **1**, **2** or **3** is not significantly different from that caused by octopamine alone, whereas a combination of 100 μM of 5-hydroxytryptamine and 100 μM of octopamine or any of

TABLE 2
Effect of Dihydrooxadiazines on Cyclic AMP Production in Nervous System Homogenates of the American Cockroach

Compound[a]	*Cyclic AMP production (pmol min^{-1} mg^{-1} protein)*
4	1111 (±55)[b]
2	1023 (±62)[b]
5	829 (±16)[b]
1	768 (±53)[b]
6	762 (±3)[b]
3	724 (±63)[b]
7	686 (±20)[b]
9	559 (±66)[b]
8	550 (±8)[b]

[a] All compounds were used at 10 μM. Cyclic AMP levels of the control, octopamine-, DCDM- and 5-hydroxytryptamine-treated homogenates were 418 (±4), 2366 (±38), 1710 (±167) and 608 (±24) pmol min^{-1} mg^{-1} protein, respectively.
[b] Significantly different from control at $P < 0.05$ ($n = 8 \pm$ SD), one-tailed pair Student's *t*-test.

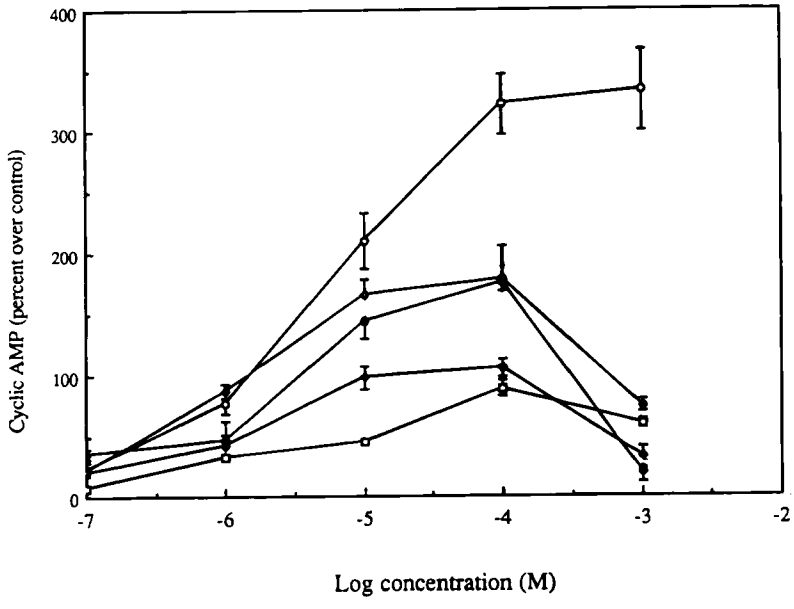

Fig. 2. Effect of different concentrations of (—○—) octopamine, dihydrooxadiazines (—◇—) **1**, (—●—) **2**, (—◆—) **3** and (—□—) 5-hydroxytryptamine on cyclic AMP production in nervous system homogenates of the American cockroach. Results are means (± SD) as % over control for two experiments with three replicates for each concentration. The control level was 525 (± 10) pmoles cyclic AMP min^{-1} mg^{-1} protein.

TABLE 3
Additivity Studies on Cyclic AMP Production in Cockroach Nervous System Homogenates Using Octopamine, 5-Hydroxytryptamine and Dihydrooxadiazines **1–3**

Treatment[a]	*Cyclic AMP production*[b] (*pmol min^{-1} mg^{-1} protein*)
Control	440 (±82)a
Octopamine	2673 (±57)b
5-Hydroxytryptamine	833 (±41)c
1	1225 (±61)d
2	1216 (±116)d
3	794 (±75)c
Octopamine + 5-hydroxytryptamine	3476 (±142)e
Octopamine + 1	2397 (±96)b
Octopamine + 2	2457 (±43)b
Octopamine + 3	2468 (±116)b
5-Hydroxytryptamine + 1	1975 (±69)f
5-Hydroxytryptamine + 2	1991 (±89)f
5-Hydroxytryptamine + 3	1593 (±52)g

[a] All compounds were tested at 100 μM. Results are means (±SD) for three experiments each performed in triplicate.
[b] Means not followed by a common letter are significantly different at the 5% level of the LSD after ANOVA.

dihydrooxadiazines **1**, **2** or **3** is significantly different from octopamine alone.

It was of interest to determine whether the elevation of cyclic AMP levels by dihydrooxadiazine compounds could be blocked by antagonists that are known to block octopamine-mediated elevation of cyclic AMP production. The results shown in Table 4 indicate that the octopaminergic antagonists mianserin, cyproheptadine, phentolamine and gramine inhibit the dihydrooxadiazine- and octopamine-mediated elevation of cyclic AMP production with the same rank-order abilities.

In order to prove that dihydrooxadiazine-induced increases in cyclic AMP production cause functional changes in cockroach nervous system cells, protein phosphorylation patterns were studied after incubating the homogenate with γ-[^{32}P]ATP in the presence and absence of dihydrooxadiazine **1**, octopamine and 8-Br-cyclic AMP. The resulting autoradiogram (Fig. 3) of labelled phosphoproteins indicates that dihydrooxadiazine **1**, octopamine and 8-Br-cyclic AMP stimulate endogenous phosphorylation. The molecular masses of the major protein bands were 14, 45, 55 and 205 kDa. The increase in endogenous protein kinase activity resulted in a decrease in overall [^{32}P]-phosphorylation as confirmed by densitometric observation (Table 5). These results demonstrate that dihydrooxadiazine **1**, octopamine and 8-Br-cyclic AMP increase endogenous phosphorylation of total proteins in homogenates of the cockroach nervous system.

The possible involvement of octopaminergic receptors in dihydrooxadiazine-mediated effects was investigated further by examining the influence of octopamine

TABLE 5
Effects of Octopamine, Dihydrooxadiazine **1** and 8-Br-cyclic AMP on Endogenous Phosphorylation of Total Proteins in Homogenates of the Cockroach Nervous System[a]

Treatment	*Levels of protein phosphorylation*[b] (*% of control*)
Octopamine	75 (±4)ab
1	83 (±5)b
8-Br-cyclic AMP	69 (±3)a

[a] All compounds were tested at 100 μM. The data were obtained by densitometric scanning of autoradiograms of SDS-polyacrylamide gel electrophoresis and are expressed as relative intensities in % of total lane intensity of control (=100).
[b] The results of two densitometric measurements of two independent experiments are expressed as means (±SD). Means not followed by a common letter are significantly different from each other at the 5% level of the LSD after ANOVA.

TABLE 4
Effects of Various Antagonists on Octopamine- and Dihydrooxadiazine-Mediated Elevation of Cyclic AMP Production in Homogenates of the Cockroach Nervous System

	Cyclic AMP level (pmol min^{-1} mg^{-1} protein)[a]			
Antagonist	*Octopamine*	*1*	*2*	*3*
—	2389 (±109)	1139 (±56)	1110 (±66)	785 (±32)
Mianserin	449 (±26)[b]	552 (±29)[c]	499 (±24)[c]	444 (±22)[c]
Cyproheptadine	464 (±11)[b]	575 (±22)[c]	723 (±28)	437 (±26)[c]
Phentolamine	498 (±24)[b]	591 (±32)[c]	746 (±44)	560 (±34)
Gramine	662 (±22)[b]	668 (±23)[c]	957 (±47)	672 (±48)

[a] All compounds were used at 100 μM. Basal level of cyclic AMP = 411 (±67) pmol min^{-1} mg^{-1} protein.
[b] Significant antagonism at $P = 0{\cdot}001$.
[c] Significant antagonism at $P < 0{\cdot}001$ ($n = 8 \pm$ SD).

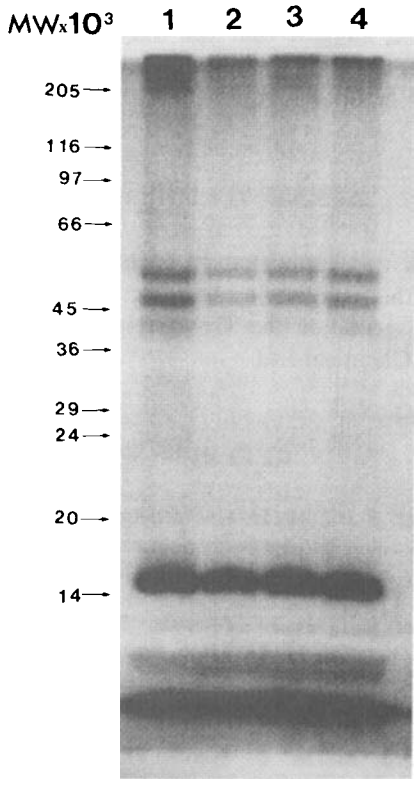

Fig. 3. Effect of 8-Br-cyclic AMP, octopamine and dihydrooxadiazine **1** on endogenous phosphorylation of specific proteins in homogenates of the nervous system of the American cockroach. Lanes shown are: Control (lane 1), 1×10^{-4} M 8-Br-cyclic AMP (lane 2), 1×10^{-4} M octopamine (lane 3) and 1×10^{-4} M dihydrooxadiazine **1** (lane 4). The figure shows an autoradiograph of SDS-polyacrylamide gel electrophoresis. Note that the proteins were first phosphorylated (nonradioactive) using endogenous protein kinase, and second, the remaining unphosphorylated proteins were phosphorylated using γ-[^{32}P]ATP and exogenously added protein kinase. Therefore, the increase in activity of the endogenous protein kinases on a given protein is expressed as the decrease in intensity of a corresponding protein band on the electrophoretogram.

and 4-Br-PDHO (**1**) on the action of proctolin on oviducal muscles in the locust (Fig. 4). Proctolin induced a large increase in contraction amplitude whereas octopamine inhibited proctolin-induced contractions. The octopamine antagonist, phentolamine, by itself had no effect on contractions of the oviduct; however, phentolamine blocked the inhibitory effects of octopamine on proctolin-induced contractions. 4-Br-PDHO (**1**) caused an effect similar to that caused by octopamine in modulating proctolin-induced contractions and its effect was also blocked by phentolamine.

4 DISCUSSION

Dihydrooxadiazines are conformationally restricted analogs of octopamine[10] and, therefore, may be expected to interact with octopamine-binding sites in biological preparations. Strong support for this proposal is provided by the results reported in the present study.

[^{3}H]DHE has been used as a ligand for monoamine-binding sites in *Drosophila* head preparations[13] and the ability of several dihydrooxadiazines to displace [^{3}H]DHE from its binding sites in homogenates of the cockroach nervous system suggests that these compounds are interacting with monoaminergic receptors. The IC_{50} values determined for dihydroergocryptine and phentolamine in the present study are similar to those reported by Dudai and Zvi[13] and serve to validate the current findings. The most effective dihydrooxadiazines in displacing [^{3}H]DHE are compounds **1**–**3** and **6** with IC_{50}s in the μM range.

The demonstration that all tested dihydrooxadiazine compounds, octopamine, 5-hydroxytryptamine and DCDM stimulate cyclic AMP production in nervous system homogenates suggests that dihydrooxadiazines may be agonists of receptors that are coupled to the adenylate cyclase system, a proposal which is consistent with findings in two-spotted spider mite homogenates.[12] However, the dihydrooxadiazine-mediated increases in cyclic AMP levels are less than those caused by octopamine or DCDM, thus the compounds may be partial, rather than full agonists of the aminergic receptor(s) as reported for formamidines.[5] Dihydrooxadiazines show

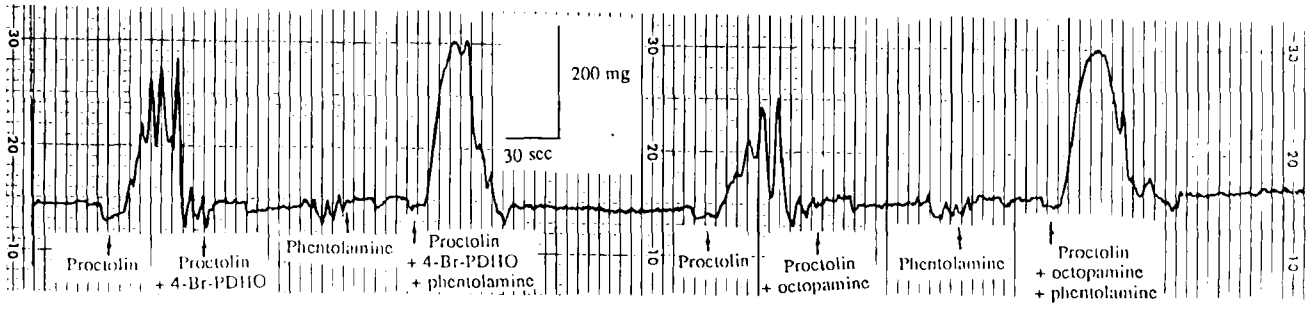

Fig. 4. Effect of 4-Br-PDHO (compound **1**) and octopamine in modulating proctolin-induced increases in the amplitude and frequency of contractions of the oviducal muscles in the locust. The preparations were exposed to saline solutions of proctolin (1×10^{-9} M), proctolin (1×10^{-9} M) + octopamine (1×10^{-6} M), phentolamine (1×10^{-4} M), proctolin (1×10^{-9} M) + octopamine (1×10^{-6} M) + phentolamine (1×10^{-4} M), proctolin (1×10^{-9} M) + 4-Br-PDHO (1×10^{-6} M), proctolin (1×10^{-9} M) + 4-Br-PDHO (1×10^{-6} M) + phentolamine (1×10^{-4} M) alternately washing the preparation with saline until myogenic contractions resumed.

no additivity with octopamine whereas additivity is observed with 5-hydroxytryptamine. Thus, it is possible that dihydrooxadiazines are interacting with octopamine-specific rather than 5-hydroxytryptamine-specific receptors.

[^{3}H]DHE is an effective ligand of α-adrenergic receptors in mammalian tissues[20] but, in insects, the ligand appears to bind to serotoninergic and other receptors.[13] The results of the current study show that dihydrooxadiazines are potent displacers of [^{3}H]DHE binding, thereby lending further credence to the suggestion that these compounds exert insecticidal action through perturbation of monoaminergic-receptor binding. All tested octopamine antagonists block the dihydrooxadiazine-mediated elevation of cyclic AMP in the nervous system homogenates. Moreover, compound **1** and octopamine also stimulate protein phosphorylation by activating endogenous protein kinases. The change in phosphorylation is similar to that caused by 8-Br-cyclic AMP which is able to penetrate the plasma membrane and thereby directly activate cyclic AMP-dependent protein kinases present in the cell. Such phosphorylation occurs at the same sites as those affected by exogenously added cyclic AMP-dependent protein kinase.[3,4,12] Thus the dihydrooxadiazine-induced elevation of cyclic AMP production appears to result directly in activation of an endogenous cyclic AMP-dependent protein kinase. These observations agree with a previous report that dihydrooxadiazines cause an increase in endogenous phosphorylation in two-spotted spider mite homogenates.[12] The present study suggests that the primary site of dihydrooxadiazine action is through octopamine-sensitive receptors.

Further support for a proposed octopaminergic site of action for the dihydrooxadiazines is provided by physiological studies in which the action of compound **1** in modulating the action of the peptide proctolin mimicked the action of octopamine. Furthermore, the effects of both octopamine and compound **1** on proctolin-induced contractions of the oviducal muscles are blocked by the octopamine antagonist phentolamine. Compound **1** was chosen based on its potent effect on cyclic AMP production in two-spotted spider mite homogenates.[12] These data are consistent with reports[14,21] that octopamine reduces proctolin-induced increases in the amplitude and frequency of contractions of the oviducal muscles in locusts.

In conclusion, dihydrooxadiazines inhibit binding of [^{3}H]DHE in homogenates of nervous tissue, elevate cyclic AMP production in a non-additive manner with octopamine, and demonstrate an octopamine-like action in modulating proctolin-induced contractions in locust oviducal preparations. These results, together with the structural similarity of these compounds to octopamine, suggest that the dihydrooxadiazines may exert their insecticidal and miticidal actions through binding to octopaminergic receptors.

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